QUANTITATION USING QUANTIFILER™ TRIO

A. SCOPE

The Quantifiler™ Trio Kit is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample with the use of TaqMan® quantitative real-time PCR technology.

For complete information regarding the Quantifiler™ Trio Kit, please refer to the associated user guide.

B. QUALITY CONTROL

B.1 Protective gloves, a lab coat, and a mask must be worn at all times when performing this procedure to prevent contamination.

B.2 Decontaminate the bench work area with a bleach-based cleaner, e.g. Clorox Bleach Germicidal Cleaner, before and after quantitation set up.

B.3 See DOC ID 1835 to determine reagent expiration dates.

B.4 Each new lot of Quantifiler™ Trio must undergo quality control testing prior to being used for the quantitation of DNA extracts.

Quality Control Testing: A standard curve using the reagents from the new lot of Quantifiler™ Trio will be prepared along with a standard curve prepared using the previously quality control tested kit. At least two non-probative samples (including at least one male sample) and a negative control will be quantitated using the new reagents and the old reagents. The samples quantitated using the new reagents will be analyzed using the new standard curve and the samples quantitated using the old reagents will be analyzed using the old standard curve. Samples used as part of the quality control testing should have a concentration of less than 50 ng/µL. Samples may be diluted to achieve a concentration of less than 50 ng/µL. Results from the same non-probative sample should be within 50% of each other. For example, if a sample is 20 ng/µL when quantitated using the old reagents and standard curve then the same sample quantitated using the new reagents and standard curve should be no less than 10 ng/µL and no greater than 30 ng/µL. The "Quant Kit QC Calculator" (DOC ID 15365) should be used for this calculation. The negative control must be free from contaminants.

B.5 Each new lot of TE^{-4} must undergo quality control testing prior to being used to dilute DNA extracts.

Quality Control Testing: A known sample will be diluted with the TE^{-4} undergoing quality control testing along with an amplification negative control containing 17.5□L of TE^{-4}. These samples will be carried through autosomal amplification and electrophoresis. The TE^{-4} will pass quality control testing when a good quality DNA profile with the correct results is obtained for the diluted sample, as described in the GlobalFiler interpretation guidelines (DOC ID 12628) and the amplification negative control is free from contaminants. The quality control data will be placed into the critical reagent binder.

B.6 At least one negative and one positive quantitation control must be quantitated on each quantitation plate.

B.7 Optical plates should be kept in the appropriate base at all times during plate setup and centrifugation. This limits the amount of debris introduced into the AB 7500 instrument and prevents damage to the plate wells that may interfere with the optical readings.

B.8 Quantitation setup must be performed in the pre-amplification room. Do not bring quantitation trays into the post amplification room.

C. SAFETY

C.1 Protective gloves, a lab coat, and a mask must be worn at all times when performing this procedure. Additionally, eye protection (e.g. safety glasses or a face shield) must be worn if this procedure is performed outside of a hood.

C.2 All appropriate SDS sheets must be read prior to performing this procedure.

C.3 Treat all biological specimens as potentially infectious.

C.4 Distinguish all waste as general, biohazard, or sharps and discard appropriately. The heat block of the AB 7500 can become very hot. Be careful not to touch the heating surfaces during plate loading and unloading.

D. REAGENTS, STANDARDS, AND CONTROLS

D.1 Quantifiler™ Trio Kit (Applied Biosystems by Thermo Fisher Scientific)

D.2 Bleach-based cleaner, e.g. Clorox Bleach Germicidal Cleaner (Decontamination)

D.3 70% Reagent Alcohol (Decontamination)

D.4 TE^{-4} (10mM Tris-HCl- 0.1mM EDTA, 1L)
Add 10mL 1 M Tris-HCl, pH 8 and 150µl 0.5 M EDTA to 990mL deionized water. Store at room temperature.

E. EQUIPMENT & SUPPLIES

E.1 Equipment

E.1.1 AB 7500 Real-Time PCR instrument and HID RT analysis software
E.1.2 Microcentrifuge
E.1.3 Pipettes
E.1.4 Vortexer
E.1.5 96 well plate centrifuge

E.2 Supplies

E.2.1 Kimwipes
E.2.2 Sterile aerosol-resistant tips
E.2.3 Microcentrifuge tubes racks
E.2.4 AB optical 96-well plates
E.2.5 AB optical adhesive covers
E.2.6 Adhesive seal applicator
E.2.7 96-well plate base
E.2.8 Disposable gloves
E.2.9 Scrubs
E.2.10 Lab coat
E.2.11 Mask
E.2.12 Eye protection (e.g. safety glasses, face shield)
E.2.13 Quant Trio Sample Sheet and Plate Map

F. PROCEDURE

F.1 Create human DNA quantitation standards by preparing the following dilution series with the 100 ng/µL DNA Standard in the Quantifiler Trio kit (volumes may be adjusted accordingly to allow for larger volumes of standards). Standards may be stored for up to 2 weeks at 2°C to 8°C

- Remove the Human DNA Quantitation Standard and allow it to thaw
- Label five clear sterile microcentrifuge tubes as Standard 1 through 5
- Add 10 uL of Dilution Buffer to the tube labeled Std.1. Aliquot 90 uL of Trio Dilution Buffer into tubes labeled Std. 2 through Std. 5. Applied Biosystems recommends changing pipet tips between each tube
- Briefly vortex the Human DNA Standard provided in the kit and pulse spin. Add 10 uL of Human DNA Standard to the tube labeled Std. 1 and mix thoroughly
- Add 10 uL of Std. 1 to the Std. 2 tube. **Vortex and pulse spin**

- Add 10 uL of Std. 2 to the Std. 3 tube. **Vortex and pulse spin**
- Continue the serial dilution through Std.5

If the dilution steps are performed as described, the DNA Standard tubes will have the following DNA concentrations:

DNA Standard	Concentration (ng/µL)
Std 1	50.0
Std 2	5.0
Std 3	0.5
Std 4	0.05
Std 5	0.005

F.2 Prior to master mix preparation, add 1 uL of Quantifiler Automation Enhancer to each 1000 uL PCR reaction mix tube and gently vortex

F.3 Thaw the Primer Mix completely. Vortex 3 -5 seconds and centrifuge briefly. Only remove the amount of Primer Mix needed for the number of reactions to reduce freeze/thawing

F.4 Determine the number of samples (evidence and standards) to be quantitated (the quant trio sample sheet and plate map template (that is also embedded in to the DNA Analysis Workbook) should be used to aid in this and the 7500 import step) and prepare the master mix by combining the following reagents in a microcentrifuge tube (or 15 mL conical tube for larger plates). Vortex 3 – 5 seconds and centrifuge briefly:

- 8 µL Primer Mix x N + 2 (where N is the total number of samples)
- 10 µL PCR Reaction Mix x N + 2 (where N is the total number of samples)

Additional reactions are included in the master mix to account for loss during reagent transfer. To minimize this effect, it is recommended to change tips frequently. Larger runs will need more additional reactions to account for larger loss

F.5 Add 18 µL of the PCR mix to each reaction well. Do not place the plate directly on the counter. Use a plate holder to prevent anything from adhering to the bottom of the plate wells. It is recommended to change tips frequently to optimize accurate pipetting

F.6 Add 2 µL of each standard, sample and blank control to the appropriate wells to bring total volume to 20 µL. Each 96-well quantitation plate will include two replicates of five DNA standards and one blank control

F.7 After all samples and standards have been aliquoted in the 96-well reaction plate, place an optical adhesive cover over the plate and seal with a rubber spatula. Avoid touching the optical cover and plate wells

F.8 Continue to PCR room for the remainder of the lab work

F.9 Spin the 96-well plate in a centrifuge at 3000 rpm for 20 seconds to remove bubbles and ensure all liquid is at the bottom

F.10 In the post-amp laboratory, turn on (or wake from sleep) the laptop connected to the 7500 instrument. Turn on the 7500 instrument and allow it to finish its starting cycle (steady green power light) before launching the 7500 HID software v 1.3. Log in as "guest"

F.11 From Home Screen (or File menu) choose experiment to run (Trio). Using the HID software, confirm the following are correct: 7500 (96 wells), Quantitation (HID standard curve) and Taqman reagents

F.12 After the template is launched, either manually enter the sample details, or select Import and Browse sample setup from the File menu.

F.13 Ensure your quant plate set up file is saved on a secure USB drive and insert this into the 7500 laptop

F.14 Locate the relevant saved file and highlight the file to import.

F.15 Input the run name in the Experiment Name line

F.16 Click on the Plate Setup button and Assign Targets and Samples tab. Verify the plate map is correct and targets have been assigned to all standards and samples

Sample	Detector	Reporter	Quencher	Task	Quantity
DNA Standard	Quantifiler Trio Large Autosomal	ABY	QSY	Standard	50†
	Quantifiler Trio Small Autosomal	VIC	MGB	Standard	50†
	Quantifiler Trio Male	FAM	MGB	Standard	50†
	IPC Quantifiler Trio	JUN	QSY	Unknown	NA
Unknown	Quantifiler Trio Large Autosomal	ABY	QSY	Unknown	NA
	Quantifiler Trio Small Autosomal	VIC	MGB	Unknown	NA
	Quantifiler Trio Male	FAM	MGB	Unknown	NA
	IPC Quantifiler Trio	JUN	QSY	Unknown	NA

† = a different numerical value will be displayed depending on the DNA standard (i.e. 50 ng/µL - 5 pg/µL)

F.17 When the template has been successfully imported, verify that the thermal cycler parameters in Run Method are as follows for Quantifiler Trio; the cycling stage has 40 cycles:

- Holding Stage: 95C for 2 min
- Cycling Stage 1: 95C for 9 sec
- Cycling Stage 2: 60C for 30 sec

F.18 Open the door of the 7500 instrument by gently pushing on the depression on the front of the instrument. Place the plate in the 7500 ensuring the orientation is correct (notched corner of plate in upper right corner of tray). Close tray by gently pushing on the depression on the front

F.19 In the top right of the software screen, click green "Start Run" box. A dialog box opens and asks you to save. Click "save", and locate the following folder to save the .eds file in:
Computer/C:/Applied Biosystems/7500/experiments/Quant Trio Data/20xx

F.20 A "Run status" window will open displaying a status of "running". Once the instrument is running the orange "in use" light will flash.

G. INTERPRETATION GUIDELINES

G.1. Look at the QC summary for any flags. Results can be viewed under the Results Tab. The Plate tab will display the quantitation results and can be used to omit wells by double clicking on the well and selecting "Omit Well". The other tabs (Spectra, Component, Amplification Plot and Standard Curve) provide additional data that may be useful in troubleshooting background or amplification problems

G.2. The Component tab displays the response to each detector during the course of the run. The detectors displayed are as follows for Quantifiler Trio: Mustang Purple as passive reference, JUN for IPC, ABY for Large Autosomal, VIC for Small Autosomal and FAM for male DNA

G.3. Check the Standard Curve by selecting Small Autosomal, Large Autosomal or Male in the Detector drop-down list to evaluate the quality of the results according to the values below. If the values fall outside these ranges, the analyst will decide whether the plate should be re-prepared and re-quantitated (if sufficient material remains), or appropriate adjustments made to subsequent amplification input amounts:

R2 value: this measures the closeness of fit between the standard curve regression line and the individual CT data points of the standards. A value of 1.00 indicates a perfect fit; for analysis, the R2 value should be > 0.98

Slope: this indicates the PCR amplification efficiency for the assay. A slope close to - 3.3 indicates optimal efficiency. The range and average of standard-curve slope values should be:

- Small Autosomal (SA): typical slope range -3.0 to – 3.6 (average -3.3)
- Large Autosomal (LA): typical slope range -3.1 to – 3.7 (average -3.4)
- Y target: typical slope range -3.0 to – 3.6 (average -3.3)

G.4. If the slope falls outside these ranges, examine the curve points to see if one of the standards should be omitted as outliers could result from pipetting errors, etc. When viewing results in the "view plate layout" view, right click on the well to omit. Select "well" or specific target within well (if metrics passed for other targets within well). Data will need to be reanalyzed

If more than one well needs to be removed to get the slope to fall within the ranges, the DNA Technical Leader should be consulted prior to continuing as there may be bigger problems with the assay that should be addressed such as software set up, reagents or instrumental issues

G.5. Print the standard curves for the SA target, LA target and Y target showing the R2 and slopes for the case file. To do this, you must "print to pdf" the slopes from the laptop and save them on your secure USB drive. When at your desktop, save the .pdf file as a .jpeg file for importing to the DNA Analysis Workbook.

G.6. Save the results (.eds file) to the Quantifiler Data folder (see F.19) naming it appropriately

G.7. Export the results in a .txt file to the secure USB drive:

- Choose "Export"
- In "**Export Properties**" tab choose:

- "Results" data to export
- "One File"
- ".txt" file type
- your USB drive as the location to save the results
- In the "**Customize Export**" tab, ensure the following order of Results data columns are checked:
- Well; Sample Name; Sample Type; Target Name; Ct;Quantity; M:F Ratio; Degradation Index

G.8. Use the radio button on the original sample setup sheet (or DNA Analysis Workbook "Report" tab) to import the quant results, then "**Unprotect Workbook**"

G.9. Print the plate map showing analyzed data for the case file

G.10. The following is a guide for the interpretation of Quant. Trio results:

Positive results:

- Positive amplification occurs when the CT value for the target is <40. Because samples contain unknown amounts of DNA and inhibitors, a large range of CT values is possible. The IPC system template DNA is present at a consistent concentration across all reactions on a plate. Therefore, the IPC (JUN® dye) CT should be relatively constant in typical reactions. However, the presence of PCR inhibitors and/or higher concentrations of DNA can increase the IPC CT relative to the average IPC CT of the quantification standards on the same plate

Negative results:

No human DNA is detected when:

- No signal for the Small Autosomal, Large Autosomal and Y targets (VIC®, ABY® and FAM™ dyes, respectively) is detected, indicating that the human and/or male-specific targets did not amplify

- The IPC target (JUN® dye) amplifies and amplification does not appear reduced relative to the average IPC CT value for quantification standards

Complete amplification failure:

- Undetected results for all assay targets, including human and male-specific targets and the IPC target, indicates a complete failure of PCR amplification for the reaction. This could be caused by conditions such as incorrect thermal cycling or incorrect formulation of PCR reagent mix (which would affect multiple reactions or possibly the entire plate), or by severe PCR inhibition affecting individual samples. This type of result is invalid and the DNA Technical Leader will need to consider the best approach for the sample(s)

PCR inhibition:

- No amplification or weak amplification of the IPC may indicate PCR inhibition (partial or complete) in the sample. In addition, suppressed amplification of the human and/or male-specific targets can occur due to PCR inhibition. This is typically more pronounced in the large autosomal target than the small autosomal target since the large autosomal target is more susceptible to inhibitory effects

IPC results inconclusive:

- With increasing concentrations of human genomic DNA (>5 ng/µL), competition between the human and/or male-specific and IPC PCR reactions may suppress IPC amplification for that sample. This can occur independently or in combination with the effect of PCR inhibitors, yielding inconclusive results. However, samples with high DNA concentration will be diluted during STR reaction setup to meet the optimal target input amount of DNA in the STR reaction. Therefore, the effect of most inhibitors, if present in the sample, on STR kit performance is likely to be minimized

Male to Female Ratio:

- The Male DNA to Female DNA Ratio can serve as an indication of male DNA to female DNA ratio and potential profiling success (or consideration for Y-STR testing), or can help the analyst decide to target more DNA for amplification.
- The M:F ratio value is a rounded estimate (as is the entire quant reaction), and represents the approximate parts of female DNA per each part of male DNA detected. You should read the result as: for every 1 part of male DNA detected, "x" many more parts of female DNA were detected.
- Generally (comporting to our internal validation data), if you read a M:F ratio value of <1:10 then GlobalFiler amplification is advised as the best amp option; between 1:10-1:30 then either or both Y-STR's and GlobalFiler may be useful; above 1:30 indicates that a Y-STR kit is probably the best amp option for a male probative question.

Degradation Index:

- The Degradation Index can serve as an indication of level of degradation to help guide troubleshooting decisions on amplification input amount; the small autosomal (SA) target is the primary quantification target for total human genomic DNA and the large autosomal (LA) target is used mainly as an indicator of DNA degradation:

  - SA target DNA conc. (ng/µL)/ LA target DNA conc. (ng/µL)

  - <1: Typically indicates that DNA is not degraded or inhibited

  - 1 to 10: Typically indicates that DNA is slightly to moderately degraded. PCR inhibition is also possible, however not enough to significantly suppress IPC amplification. Analyst may choose a target amplification amount closer to 1.0 ng (versus 0.5) to maximize allele recovery

  - >10 or blank: Typically indicates that DNA is significantly degraded. PCR inhibition is also possible, however not enough to significantly suppress IPC amplification

H. REFERENCES

H.1 Quantifiler Trio - User Guide

H.2 Quantifiler Trio - Quick Reference

H.3 WCSO Quantifiler Trio Report